ACS OMEGA

http://pubs.acs.org/journal/acsodf

Article

E-Cigarette Flavoring Chemicals Induce Cytotoxicity in HepG2 Cells

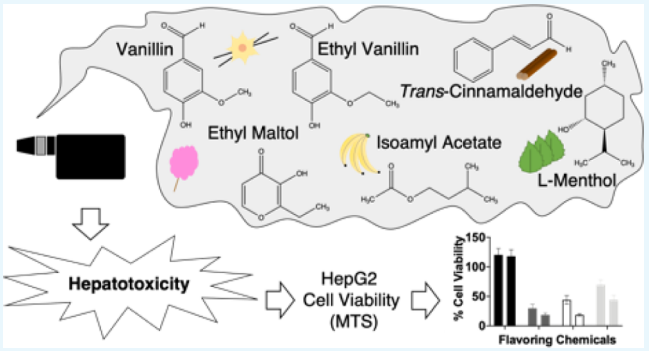
Brittany P. Rickard, Henry Ho, Jacqueline B. Tiley, Ilona Jaspers, and Kim L. R. Brouwer*

Cite This: *ACS Omega* 2021, 6, 6708−6713

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: E-cigarette-related hospitalizations and deaths across the U.S. continue to increase. A high percentage of patients have elevated liver function tests indicative of systemic toxicity. This study was designed to determine the effect of e-cigarette chemicals on liver cell toxicity. HepG2 cells were exposed to flavoring chemicals (isoamyl acetate, vanillin, ethyl vanillin, ethyl maltol, L-menthol, and *trans*-cinnamaldehyde), propylene glycol, and vegetable glycerin mixtures, and cell viability was measured. Data revealed that vanillin, ethyl vanillin, and ethyl maltol decreased HepG2 cell viability; repeated exposure caused increased cytotoxicity relative to single exposure, consistent with the hypothesis that frequent vaping can cause hepatotoxicity.



1. INTRODUCTION

The use of electronic cigarettes, or e-cigarettes, and vaping have become increasingly popular in recent years. Many consider e-cigarettes to be a safe alternative to tobacco smoking; however, vaping-related hospitalizations and deaths across the U.S. have been increasing.[1] In addition to vaping-associated lung injury, the Centers for Disease Control (CDC) has suggested that liver injury may also occur.[1] In one study, 50% of hospitalized e-cigarette users exhibited elevated serum markers of liver function, including serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST);[1,2] however, the mechanism(s) of these organ-specific effects remain to be elucidated.

E-liquids are commonly used in conjunction with certain generations of e-cigarettes, and these e-liquids typically contain a propylene glycol (PG) and vegetable glycerin (VG) base, additives of flavoring chemicals, and nicotine.[3,4] One of the factors driving the appeal of e-cigarettes are the vast array of e-liquid flavorings on the market.[4−6] There are more than 7000 e-liquid flavors available, and studies examining the composition of various marketed e-liquids have found that each e-liquid solution can contain over 60 chemicals.[4,7] Additionally, constituents of e-liquids are typically not included in the products' ingredient lists, meaning that the flavoring chemicals, among other compounds, are present in undisclosed concentrations.[4,8,9] Certain e-liquid flavors like cinnamon contain cytotoxic chemicals such as cinnamaldehyde,[10] while cotton candy can contain reactive aldehydes, such as vanillin and ethyl vanillin, as well as alcohols like ethyl maltol.[9]

The toxicity of e-liquids has been studied to a limited extent in numerous models to demonstrate the potential health impacts of e-liquid constituents. For example, Bahl *et al.*[11] reported that cytotoxicity observed in human pulmonary fibroblasts and human embryonic and mouse neural stem cells exposed to various e-liquids was related to the number and concentration of flavoring chemicals present rather than the presence of nicotine. Based on these findings, systemic toxicity observed in e-cigarette users may not only be the result of nicotine but also flavoring chemicals and other additives like PG/VG or vitamin E acetate.[3,12] Since vaping already has been shown to have systemic effects that impact the immune system and vasculature,[13] examining the impact of e-liquid components on liver cells may provide a better understanding of the possible mechanisms responsible for the hepatotoxicity of e-cigarettes.

In addition to CDC reports linking e-cigarette chemicals with hepatotoxicity, El Golli *et al.*[14] reported that e-liquids without nicotine increased liver enzyme biomarkers such as AST, ALT, and alkaline phosphatase while decreasing total liver protein, hepatic glycogen rate, and cholesterol in rats. Based on these data, we hypothesize that flavoring chemicals or other e-liquid constituents can cause hepatotoxicity. In this study, the cytotoxic effects of e-cigarette chemicals, including vanillin, ethyl vanillin, ethyl maltol, L-menthol, *trans*-cinnamaldehyde, isoamyl acetate, and PG/VG, were investigated in a human liver cancer cell line (HepG2), which is a frequently used cell model for cytotoxicity experiments.[15] Short-term single and repeated exposure of these chemicals at specific concentrations was investigated to provide a better

Received: November 19, 2020
Accepted: February 18, 2021
Published: March 2, 2021

https://dx.doi.org/10.1021/acsomega.0c05639
ACS Omega 2021, 6, 6708−6713

understanding of their potential to cause cytotoxicity. These findings add to our current understanding regarding the mechanisms responsible for liver injury observed in hospitalized e-cigarette users.

2. RESULTS AND DISCUSSION

E-cigarette chemicals were formulated in Dulbecco's modified Eagle's medium (DMEM) with and without 10% fetal bovine serum (FBS) in order to determine the effect of serum, and the potential for binding to serum proteins, on e-liquid toxicity. At a concentration of 5 mM, vanillin, ethyl vanillin, and ethyl maltol decreased HepG2 cell viability compared to controls in both serum-containing and serum-free (SF) media (Figure 1). The viability of HepG2 cells was not influenced by exposure to L-menthol or *trans*-cinnamaldehyde in media with or without serum at the concentrations tested.

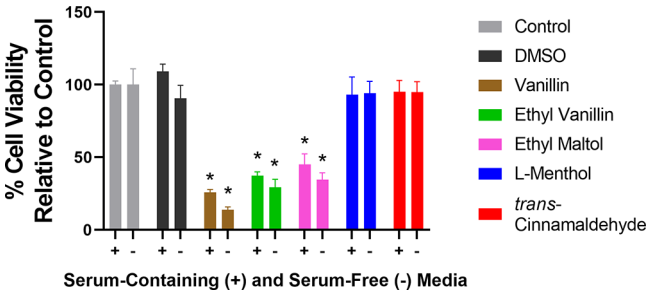


Figure 1. Cytotoxicity of flavoring chemicals in serum-containing (+) and SF (−) media. HepG2 cell viability [mean ± standard deviation (SD) expressed as a percentage of the 0.1% dimethyl sulfoxide (DMSO) control; $n = 3$ individual experiments in triplicate] after 48 h exposure to flavoring chemicals at the highest concentration tested (5 mM: vanillin, ethyl vanillin, ethyl maltol, and L-menthol; 79.4 μM *trans*-cinnamaldehyde). Significant differences between each chemical in both medium conditions versus control are denoted by * ($P < 0.05$) determined by multiple *t*-tests using the Holm-Sidak method, with $\alpha = 0.05$.

In order to explore individual concentration−response curves, each flavoring chemical was tested at four different concentrations in SF media. At the highest concentration, some flavoring chemicals (*e.g.*, vanillin, ethyl vanillin, and ethyl maltol) decreased HepG2 cell viability by over 80% after 48 h compared to the 0.1% DMSO control (Figure 2A−C). Additionally, cell viability decreased significantly in cells exposed to 5 mM ethyl maltol for 48 h compared to 24 h. After 24 h, cell viability was decreased after exposure to these chemicals at various concentrations, although to a lesser extent than after 48 h. The viability of cells exposed to L-menthol did not appear to differ over the concentration range studied (100 nM−2.5 mM), or between the 24- and 48 h exposure times (Figure 2D). When cells were exposed to *trans*-cinnamaldehyde, cell viability was decreased at 10 nM and 10 μM after 48 h; at 10 μM, cell viability was significantly decreased after 48 h compared to 24 h exposure (Figure 2E). Similar to our findings, Hua *et al.*[16] found that vanillin, ethyl vanillin, and ethyl maltol were the most toxic e-cigarette refill liquid chemicals based on their respective half-maximal inhibitory concentration (IC_{50}) values in mouse neural stem cells and BEAS-2B cells.

Since e-cigarette users are repeatedly exposed to flavoring chemicals as they vape, the cytotoxic effects of repeated exposure of these chemicals to HepG2 cells were examined. E-

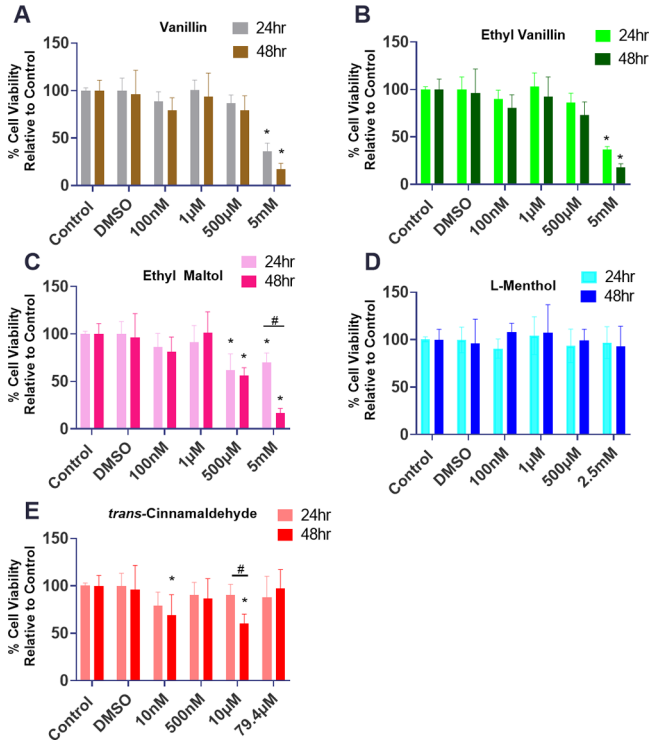


Figure 2. Cell viability after exposure to flavoring chemicals e-cigarette chemicals after 24- and 48 h exposure to HepG2 cells. Concentration−response curves [mean ± SD; ($n = 3$ individual experiments in triplicate) of (A) vanillin, (B) ethyl vanillin, (C) ethyl maltol, (D) L-menthol, and (E) *trans*-cinnamaldehyde] in SF media expressed as a percentage of the 0.1% DMSO control. Significance between 0.1% DMSO control and each chemical concentration at 24- or 48 h is denoted by * ($P < 0.05$), and significance between 24- and 48 h timepoints of each concentration is denoted by # ($P < 0.05$) determined by a two-way ANOVA followed by Tukey's multiple comparison tests for correction.

cigarette chemicals were added in serum-containing or SF media at the highest concentration to HepG2 cells every 30- or 90 min for 5 h. After 30- and 90 min repeated exposure in serum-containing media, significant decreases in cell viability compared to the 0.1% DMSO control were observed for vanillin, ethyl vanillin, and L-menthol; cell viability was also significantly decreased after 90 min repeated exposure to ethyl maltol (Figure 3A,B). Interestingly, after 30 min repeated exposure in SF media, a significant decrease in cell viability for ethyl maltol and *trans*-cinnamaldehyde, in addition to vanillin, ethyl vanillin, and L-menthol compared to the 0.1% DMSO control was observed (Figure S1A,B). Additionally, increased cytotoxicity after 30 min repeated exposure was observed for *trans*-cinnamaldehyde in SF media compared to serum-containing media. Similarly, after 90 min repeated exposure in SF media, cells exposed to *trans*-cinnamaldehyde in addition to vanillin, ethyl vanillin, ethyl maltol, and L-menthol had decreased cell viability compared to the 0.1% DMSO control (Figure S2A,B). Cytotoxicity of *trans*-cinnamaldehyde and 20:80 PG/VG was also significantly increased in SF media compared to serum-containing media after 90 min repeated exposure. Interestingly, while L-menthol exposure at 5 mM in Figure 1 did not appear to impact cell viability, 30- and 90 min repeated exposure to 5 mM L-menthol significantly decreased cell viability compared to the 0.1% DMSO control. This finding may be due to the total exposure of L-menthol, or a

https://dx.doi.org/10.1021/acsomega.0c05639
ACS Omega 2021, 6, 6708−6713

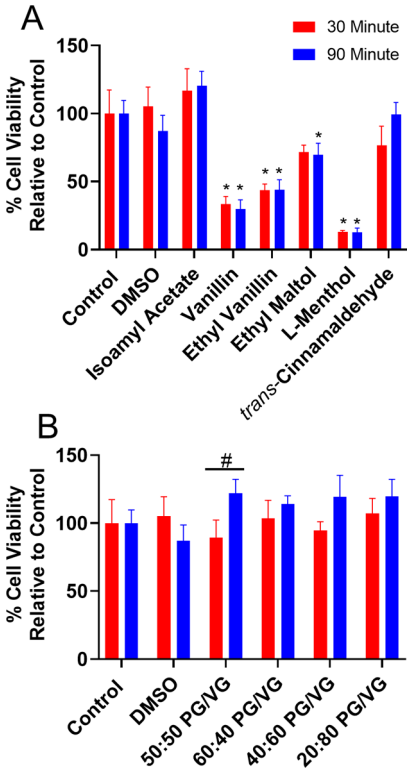


Figure 3. Cytotoxicity of e-cigarette chemicals after repeated exposure. (A) Effects of flavoring chemicals (67.3 μM isoamyl acetate, 5 mM vanillin, 5 mM ethyl vanillin, 5 mM ethyl maltol, 5 mM L-menthol, and 79.4 μM *trans*-cinnamaldehyde) on HepG2 cells after repeated exposure. (B) Effects of PG/VG mixtures (50:50, 60:40, 40:60, and 20:80) on HepG2 cells after repeated exposure. HepG2 cells were exposed to each flavoring chemical or PG/VG mixture in serum-containing media every 30 (red) or 90 (blue) min for 5 h, followed by incubation with each flavoring chemical or PG/VG mixture for an additional 43 h (total exposure time = 48 h). Data are presented as mean ± SD (n = 3 individual experiments in triplicate). Significance between control and each chemical is denoted by * (P < 0.05), and significance between 30- and 90 min repeated exposure for each chemical is denoted by # (P < 0.05) determined by a two-way ANOVA followed by Tukey's multiple comparison tests for correction.

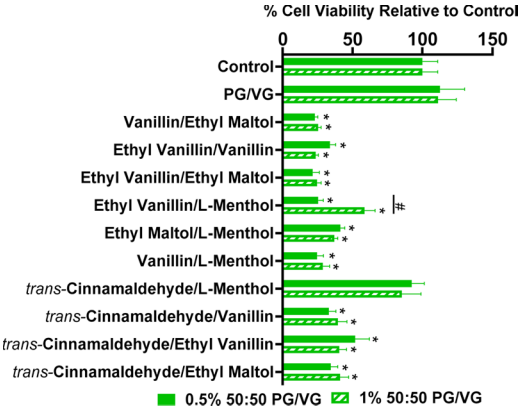


Figure 4. Cytotoxicity of e-cigarette chemicals after exposure to cocktails. Cytotoxicity of flavoring chemical cocktails with 0.5% and 1% 50:50 PG/VG in SF media after 48 h exposure to HepG2 cells relative to 0.1% DMSO plus 0.5 or 1% 50:50 PG/VG control. Data are presented as mean ± SD (n = 3 individual experiments in triplicate). Significance between control and each cocktail is denoted by * (P < 0.05), and significance between the PG/VG composition of each cocktail (0.5 *vs* 1%) is denoted by # (P < 0.05) determined by a two-way ANOVA followed by Tukey's multiple comparison tests for correction.

formed metabolite, which would be higher after repeated exposure compared to a single exposure. Repeated exposure experiments were performed in serum-containing and SF media in order to account for potential protein-binding effects; however, only the data generated in serum-containing media are included in Figure 3A,B, which would be more relevant to the *in vivo* situation.

Although flavoring chemicals alone decreased HepG2 cell viability, e-liquids rarely are composed exclusively of one flavoring chemical. Instead, e-liquids are mixtures of flavoring chemicals combined with PG/VG and other additives.[3] Therefore, the effects of flavoring chemicals combined with PG/VG (referred to as cocktails) were examined in order to simulate the impact of e-liquids in HepG2 cells. The results of these experiments indicated that cocktails decreased HepG2 cell viability compared to 0.5 or 1% PG/VG controls (Figure 4). Specifically, all cocktails examined (vanillin/ethyl maltol, ethyl vanillin/vanillin, ethyl vanillin/ethyl maltol, ethyl vanillin/L-menthol, ethyl maltol/L-menthol, vanillin/L-menthol, *trans*-cinnamaldehyde/vanillin, *trans*-cinnamaldehyde/ethyl vanillin, and *trans*-cinnamaldehyde/ethyl maltol) showed significantly decreased cell viability compared to PG/VG controls with the exception of *trans*-cinnamaldehyde/L-menthol.

Similar to our findings that PG/VG alone does not decrease HepG2 cell viability (Figures 3B and 4), others have found that PG and VG alone do not cause cytotoxicity in neonatal, fetal, or adult ovine pulmonary artery smooth muscle cells.[17] However, previous studies have reported that acetals can be formed between PG/VG and certain flavoring chemicals, such as vanillin and ethyl vanillin, thereby enhancing their toxic effects.[18,19] When HepG2 cells were exposed to cocktails of flavoring chemicals and PG/VG, cell viability decreased significantly for all combinations compared to PG/VG controls except *trans*-cinnamaldehyde/L-menthol. Studies examining the effects of menthol and cinnamaldehyde in lung cell culture models have found that transient receptor potential cation channel subfamily M member 8 (TRMP8) and transient receptor potential ankyrin 1 (TRPA1), respectively, play significant roles in mediating cytotoxicity.[20−22] Since HepG2 cells lack TRPA1 expression,[23] this may explain the general lack of observed cytotoxicity with *trans*-cinnamaldehyde. Also, studies have shown that L-menthol and *trans*-cinnamaldehyde have the ability to counteract each other when administered dermally together or sequentially, which could explain our findings.[24] HepG2 cell viability was significantly lower when the ethyl vanillin/L-menthol cocktail was formulated with 0.5% PG/VG compared to 1% PG/VG, which warrants further investigation. These data clearly demonstrate the ability of certain flavoring chemicals and PG/VG cocktails to decrease HepG2 cell viability.

Unfortunately, serum concentrations of e-cigarette chemicals have not been measured in humans after vaping. Concentrations that have been studied previously *in vitro* range from picomolar to millimolar.[19] Based on recommendations from scientists working in the field (personal communication), concentrations of the e-cigarette chemicals selected for the present studies covered a wide range. An important next step is

https://dx.doi.org/10.1021/acsomega.0c05639
ACS Omega 2021, 6, 6708−6713

to measure relevant concentrations of e-cigarette chemicals in biological fluids of e-cigarette users.

3. CONCLUSIONS

Overall, these studies suggest that flavoring chemicals and cocktails decrease viability of a commonly used liver cell model. Based on the data presented, flavoring chemicals of particular interest for future studies are vanillin, ethyl vanillin, ethyl maltol, and L-menthol. While PG/VG combinations alone did not appear to decrease HepG2 cell viability, they may contribute to the observed cytotoxicity with the cocktails and warrant further examination. It is well documented that e-cigarettes have the potential to cause systemic toxicity, including liver injury. As e-liquids containing flavoring chemicals have grown in popularity, especially among youth, it is clear that additional research is needed to understand the mechanisms of toxicity and the chemical combinations that have the greatest liability for hepatotoxicity.

4. EXPERIMENTAL SECTION

4.1. Materials. Working solutions of 5 M vanillin (Sigma #W310727; ≥97% purity), ethyl vanillin (Sigma #W246409; ≥98% purity), ethyl maltol (Sigma #W348708; ≥99% purity), and L-menthol (Sigma #W266590; ≥99% purity) were prepared in DMSO. In addition, working solutions of flavoring chemicals including *trans*-cinnamaldehyde (79.4 mM, Sigma #W228605; ≥98% purity) and isoamyl acetate (67.3 mM, Sigma #W205532; ≥97% purity) were prepared in DMSO. PG (Fisher #P355-1; >99% purity)/VG (Sigma #G2289; >99% purity) solutions were prepared at the following ratios: 50:50, 60:40, 40:60, and 20:80 in DMSO (0.1% of solution).

4.2. Cell Culture. HepG2 cells [American type culture collection (ATCC)] were cultured in two conditions: DMEM (Gibco #11885-076), 1% Penicillin/Streptomycin (Gibco #15140-122), and (A) with 10% FBS (Millipore #TMS-013-B) and (B) without FBS. HepG2 cells were authenticated by short tandem repeat analysis at the Vironomics Core at the UNC School of Medicine. For cytotoxicity experiments, HepG2 cells were seeded in 96-well plates at 20,000 cells/well and allowed to grow for 48 h prior to exposure to e-cigarette chemicals. For the purpose of this manuscript, cytotoxicity is defined as a >30% decrease in HepG2 cell viability as a result of exposure to e-cigarette compounds.

In order to minimize the potential for e-cigarette chemicals to vaporize and affect the viability of adjacent wells, each chemical was separated by two rows of wells (vapor control wells) filled with media only. This approach was based on previous studies[10] and discussion with other scientists in the field (personal communication).

4.3. Concentration−Response Experiments. Treatment concentrations of flavoring chemicals for concentration−response experiments were 100 nM, 1 μM, 500 μM, and 2.5 mM for L-menthol and 100 nM, 1 μM, 500 μM, and 5 mM for vanillin, ethyl vanillin, and ethyl maltol in media at room temperature. The concentration of L-menthol was lowered from 5 to 2.5 mM for these experiments due to precipitation in media at room temperature; however, for serum/SF and repeated exposure experiments, L-menthol was successfully exposed to the cells at 5 mM without precipitation in warm media. Since isoamyl acetate (6.73 M) and *trans*-cinnamaldehyde (7.94 M) stock solutions were in the liquid form, working solutions were prepared in 99% DMSO, resulting in concentrations of 67.3 and 79.4 mM, respectively. Isoamyl acetate and *trans*-cinnamaldehyde were subsequently exposed to the cells at 10 nM, 500 nM, and 10 μM and 1000-fold less than the working solutions to completely remove interference from DMSO (isoamyl acetate, 67.3 μM; *trans*-cinnamaldehyde, 79.4 μM).

4.4. Repeated Exposure Experiments. For experiments involving repeated exposure, flavoring chemicals were prepared at the highest dosing concentrations (79.4 μM *trans*-cinnamaldehyde, 67.3 μM isoamyl acetate, and 5 mM L-menthol, vanillin, ethyl vanillin, and ethyl maltol) in warm media. PG/VG was formulated in exposure media at 0.1% 50:50, 60:40, 40:60, or 20:80. Cells were exposed to media containing each respective e-cigarette chemical (exposure media) at 0 h. After 30- or 90 min, the original exposure media was aspirated. After aspiration, fresh exposure media was added to each well for another 30- or 90 min. After 30- or 90 min, exposure media was aspirated again, and this process was repeated every 30- or 90 min for a total exposure time of 5 h. At 5 h, exposure media was aspirated from each well and fresh exposure media was added for 43 h, for a total exposure time of 48 h.

4.5. Cocktails. When preparing cocktails of flavoring chemicals and PG/VG, 50:50 PG/VG was exposed to cells as 0.5 and 1% of solution in SF DMEM with 0.1% DMSO. In these mixtures, two flavoring chemicals were added at one-half of the maximum dosing concentration for each chemical (vanillin, ethyl vanillin, ethyl maltol, and L-menthol at 2.5 mM and *trans*-cinnamaldehyde at 39.7 μM). Mixtures were exposed to the cells in SF media for 48 h.

4.6. MTS Assay. After 24 h, 48 h, and repeated exposure of every 30- and 90 min for 5 h to e-cigarette chemicals, cells were examined for cytotoxicity using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega #G3582; Madison, WI), according to the manufacturer's instructions. Briefly, tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium], inner salt (MTS) was added to the media and absorption (at 490 nM) was measured after 2.5 h. DMEM and 0.1% DMSO with/without 10% FBS were used as negative controls. Triton-X (1%) was used as a positive control, and toxicity (up to 95%) was observed (data not shown). The purpose of this assay is to assess cell metabolic activity as an indicator of mitochondrial function and cell viability.

4.7. Data and Statistical Analyses. GraphPad Prism 8.4.3 was used for statistical analyses and comparisons of data sets. Multiple *t*-tests, using the Holm-Sidak method where $\alpha = 0.05$, or a two-way ANOVA followed by Tukey's multiple comparison tests for correction were used for data analysis, as designated in the figure legends. Data are shown as mean ± SD ($n = 3$ individual experiments in triplicate for each concentration). Similar to the tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), or MTT, assay,[25] variability may also be a concern for the MTS assay; therefore, a mean difference of >30% was used as an arbitrary cut-off value for significance.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c05639.

https://dx.doi.org/10.1021/acsomega.0c05639
ACS Omega 2021, 6, 6708−6713

Cytotoxicity of e-cigarette chemicals after 30- and 90 min repeated exposure in varying medium conditions (PDF)

AUTHOR INFORMATION

Corresponding Author

Kim L. R. Brouwer − *Division of Pharmacotherapy and Experimental Therapeutics, UNC Eshelman School of Pharmacy and Curriculum in Toxicology & Environmental Medicine, UNC School of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7569, United States;* orcid.org/0000-0003-1945-4929; Phone: (919) 962-7030; Email: kbrouwer@unc.edu; Fax: (919) 962-0644

Authors

Brittany P. Rickard − *Curriculum in Toxicology & Environmental Medicine, UNC School of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7569, United States;* orcid.org/0000-0002-6112-1290

Henry Ho − *Division of Pharmacotherapy and Experimental Therapeutics, UNC Eshelman School of Pharmacy, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7569, United States*

Jacqueline B. Tiley − *Division of Pharmacotherapy and Experimental Therapeutics, UNC Eshelman School of Pharmacy, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7569, United States*

Ilona Jaspers − *Curriculum in Toxicology & Environmental Medicine, UNC School of Medicine and Center for Environmental Medicine, Asthma, and Lung Biology, UNC School of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7569, United States;* orcid.org/0000-0001-8728-0305

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c05639

Author Contributions

All authors contributed to, and have approved, the final version of this manuscript.

Funding

This research was supported by the National Institutes of Health, National Institute of Environmental Health Sciences (T32 ES007126).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors would like to acknowledge the Jaspers Laboratory for providing chemicals and information about dosing concentrations and chemical stability.

ABBREVIATIONS

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATCC, American type culture collection; CDC, Centers for Disease Control and prevention; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HepG2, human liver cancer cell line; IC_{50}, half-maximal inhibitory concentration; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); PG, propylene glycol; SD, standard deviation; SF, serum-free; STR, short tandem repeat; TRMP8, transient receptor potential cation channel subfamily M member 8; TRPA1, transient receptor potential ankyrin 1; VG, vegetable glycerin

REFERENCES

(1) Siegel, D. A.; Jatlaoui, T. C.; Koumans, E. H.; Kiernan, E. A.; Layer, M.; Cates, J. E.; Kimball, A.; Weissman, D. N.; Petersen, E. E.; Reagan-Steiner, S.; Godfred-Cato, S.; Moulia, D.; Moritz, E.; Lehnert, J. D.; Mitchko, J.; London, J.; Zaki, S. R.; King, B. A.; Jones, C. M.; Patel, A.; Delman, D. M.; Koppaka, R.; Lung Injury Response Clinical Working, G.; Lung Injury Response Epidemiology/Surveillance, G.. Update: Interim Guidance for Health Care Providers Evaluating and Caring for Patients with Suspected E-cigarette, or Vaping, Product Use Associated Lung Injury—United States, October 2019. *Morb. Mortal. Wkly. Rep.* **2019**, *68*, 919−927.

(2) Layden, J. E.; Ghinai, I.; Pray, I.; Kimball, A.; Layer, M.; Tenforde, M. W.; Navon, L.; Hoots, B.; Salvatore, P. P.; Elderbrook, M.; Haupt, T.; Kanne, J.; Patel, M. T.; Saathoff-Huber, L.; King, B. A.; Schier, J. G.; Mikosz, C. A.; Meiman, J. Pulmonary Illness Related to E-Cigarette Use in Illinois and Wisconsin—Final Report. *N. Engl. J. Med.* **2020**, *382*, 903−916.

(3) Sassano, M. F.; Davis, E. S.; Keating, J. E.; Zorn, B. T.; Kochar, T. K.; Wolfgang, M. C.; Glish, G. L.; Tarran, R. Evaluation of E-Liquid Toxicity Using an Open-Source High-Throughput Screening Assay. *PLoS Biol.* **2018**, *16*, No. e2003904.

(4) National Academies of Sciences, Engineering, and Medicine. *Public Health Consequences of E-Cigarettes*; The National Academies Press: Washington, DC, 2018; p 774.

(5) Ambrose, B. K.; Day, H. R.; Rostron, B.; Conway, K. P.; Borek, N.; Hyland, A.; Villanti, A. C. Flavored Tobacco Product Use Among US Youth Aged 12-17 Years, 2013-2014. *JAMA, J. Am. Med. Assoc.* **2015**, *314*, 1871−1873.

(6) Villanti, A. C.; Richardson, A.; Vallone, D. M.; Rath, J. M. Flavored Tobacco Product Use Among U.S. Young Adults. *Am. J. Prev. Med.* **2013**, *44*, 388−391.

(7) Herrington, J. S.; Myers, C. Electronic Cigarette Solutions and Resultant Aerosol Profiles. *J. Chromatogr. A* **2015**, *1418*, 192−199.

(8) Hahn, J.; Monakhova, Y. B.; Hengen, J.; Kohl-Himmelseher, M.; Schussler, J.; Hahn, H.; Kuballa, T.; Lachenmeier, D. W. Electronic Cigarettes: Overview of Chemical Composition and Exposure Estimation. *Tob. Induced Dis.* **2014**, *12*, 23.

(9) Tierney, P. A.; Karpinski, C. D.; Brown, J. E.; Luo, W.; Pankow, J. F. Flavour Chemicals in Electronic Cigarette Fluids. *Tob. Control* **2016**, *25*, e10−e15.

(10) Behar, R. Z.; Luo, W.; Lin, S. C.; Wang, Y.; Valle, J.; Pankow, J. F.; Talbot, P. Distribution, Quantification and Toxicity of Cinnamaldehyde in Electronic Cigarette Refill Fluids and Aerosols. *Tob. Control* **2016**, *25*, ii94−ii102.

(11) Bahl, V.; Lin, S.; Xu, N.; Davis, B.; Wang, Y.-h.; Talbot, P. Comparison of Electronic Cigarette Refill Fluid Cytotoxicity Using Embryonic and Adult Models. *Reprod. Toxicol.* **2012**, *34*, 529−537.

(12) https://www.cdc.gov/tobacco/basic_information/e-cigarettes/severe-lung-disease.html#what-we-know Date accessed: 10/01/2020.

(13) Chaffee, B. W. Electronic Cigarettes: Trends, Health Effects and Advising Patients Amid Uncertainty. *J. Calif. Dent. Assoc.* **2019**, *47*, 85−92.

(14) El Golli, N.; Dkhili, H.; Dallagi, Y.; Rahali, D.; Lasram, M.; Bini-Dhouib, I.; Lebret, M.; Rosa, J.-P.; El Fazaa, S.; Asmi, M. A.-E. Comparison Between Electronic Cigarette Refill Liquid and Nicotine on Metabolic Parameters in Rats. *Life Sci.* **2016**, *146*, 131−138.

(15) Donato, M. T.; Tolosa, L.; Gómez-Lechón, M. J. Culture and Functional Characterization of Human Hepatoma HepG2 Cells. *Methods Mol. Biol.* **2015**, *1250*, 77−93.

(16) Hua, M.; Omaiye, E. E.; Luo, W.; McWhirter, K. J.; Pankow, J. F.; Talbot, P. Identification of Cytotoxic Flavor Chemicals in Top-Selling Electronic Cigarette Refill Fluids. *Sci. Rep.* **2019**, *9*, 2782.

(17) Berkelhamer, S. K.; Helman, J. M.; Gugino, S. F.; Leigh, N. J.; Lakshminrusimha, S.; Goniewicz, M. L. In Vitro Consequences of Electronic-Cigarette Flavoring Exposure on the Immature Lung. *Int. J. Environ. Res. Public Health* **2019**, *16*, 3635.
(18) Erythropel, H. C.; Jabba, S. V.; DeWinter, T. M.; Mendizabal, M.; Anastas, P. T.; Jordt, S. E.; Zimmerman, J. B. Formation of Flavorant-Propylene Glycol Adducts With Novel Toxicological Properties in Chemically Unstable E-Cigarette Liquids. *Nicotine Tob. Res.* **2019**, *21*, 1248−1258.
(19) Hickman, E.; Herrera, C. A.; Jaspers, I. Common E-Cigarette Flavoring Chemicals Impair Neutrophil Phagocytosis and Oxidative Burst. *Chem. Res. Toxicol.* **2019**, *32*, 982−985.
(20) Lin, A.-H.; Liu, M.-H.; Ko, H.-K.; Perng, D.-W.; Lee, T.-S.; Kou, Y. R. Menthol Cigarette Smoke Induces More Severe Lung Inflammation Than Non-menthol Cigarette Smoke Does in Mice With Subchronic Exposure—Role of TRPM8. *Front. Physiol.* **2018**, *9*, 1817.
(21) Nair, V.; Tran, M.; Behar, R. Z.; Zhai, S.; Cui, X.; Phandthong, R.; Wang, Y.; Pan, S.; Luo, W.; Pankow, J. F.; Volz, D. C.; Talbot, P. Menthol in Electronic Cigarettes: A Contributor to Respiratory Disease? *Toxicol. Appl. Pharmacol.* **2020**, *407*, 115238.
(22) Lin, A.-H.; Liu, M.-H.; Ko, H.-K.; Perng, D.-W.; Lee, T.-S.; Kou, Y. R. Lung Epithelial TRPA1 Transduces the Extracellular ROS into Transcriptional Regulation of Lung Inflammation Induced by Cigarette Smoke: The Role of Influxed Ca^{2+}. *Mediators Inflammation* **2015**, *2015*, 148367.
(23) Badr, H.; Kozai, D.; Sakaguchi, R.; Numata, T.; Mori, Y. Different Contribution of Redox-Sensitive Transient Receptor Potential Channels to Acetaminophen-Induced Death of Human Hepatoma Cell Line. *Front. Pharmacol.* **2016**, *7*, 19.
(24) Andersen, H. H.; Gazerani, P.; Arendt-Nielsen, L. High-Concentration L-Menthol Exhibits Counter-Irritancy to Neurogenic Inflammation, Thermal and Mechanical Hyperalgesia Caused by Trans-Cinnamaldehyde. *J. Pain* **2016**, *17*, 919−929.
(25) van Tonder, A.; Joubert, A. M.; Cromarty, A. Limitations of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) Assay When Compared to Three Commonly Used Cell Enumeration Assays. *BMC Res. Notes* **2015**, *8*, 47.

https://dx.doi.org/10.1021/acsomega.0c05639
ACS Omega 2021, 6, 6708−6713